PROTEIN PRODUCTION METHODS AND MODIFIED CELLS FOR USE THEREIN

Background of the Invention

This invention relates the field of cell biology. More particularly, this invention relates to protein production by eukaryotic cells.

Proteins produced by eukaryotic cells can have significant therapeutic value. Such proteins may be naturally produced by the eukaryotic cell, or the eukaryotic cell may be manipulated by recombinant molecular biology techniques to produce a heterologous protein. Non-limiting examples of proteins produced, either naturally or by artifice, include erythropoietin, insulin, and factor IX.

In eukaryotic cell culture, production of protein from a cultured cell is a function of the specific activity (*i.e.*, the amount of protein produced per cell) and the total viable cell mass over the course of a bioreactor run (ICA). However, the production of protein by cultured cells is limited by cell death in the bioreactor. There are two forms of cell death, necrosis and apoptosis. Necrosis is a form of cell death that is typically due to a traumatic injury or insult to the cell. Shear forces and foaming are probable causes of necrosis in the bioreactor. Apoptosis, also known as programmed cell death, is a form of cell death where, through a variety of signaling pathways, the cell self-destructs. Examples of apoptosis stimuli include growth factor withdrawal, the limitation of various nutrients and exposure to toxins. Recent literature on the subject of cell death in the bioreactor supports the notion of apoptosis as a major contributor (Moore A *et al.*, "Apoptosis in CHO cell batch cultures: examination by flow cytometry," *Cytotechnology* **17**:1-11, 1995; Goswami J *et al.*, "Apoptosis in Batch Cultures of Chinese Hamster Ovary Cells," *Biotechn. & Bioeng.* **62**:632-640, 1999).

There is a need to identify methods for prolonging cell lifespan as a means for enhancing the cell's production of a protein, regardless of whether that protein is one naturally produced by the cell, or whether that protein is a heterologous protein to the cell.

Summary of the Invention

The invention provides methods for prolonging cell lifespan as a means for enhancing the cell's production of a protein, regardless of whether that protein is one naturally produced by the cell, or whether that protein is a heterologous protein to the

cell.

Accordingly, in one aspect, the invention provides a method for increasing production of a protein by a cell, comprising increasing expression of an anti-apoptosis gene in the cell. In certain embodiments, the cell does not express a heterologous cyclin-dependent kinase inhibitor. In particular embodiments, the cell is a human cell, a murine cell, a hamster cell, an insect cell, or an amphibian cell.

In another aspect, the invention provides a method for increasing the production of a heterologous protein by a cell, comprising increasing expression of an anti-apoptosis gene in the cell, wherein the cell does not express a heterologous cyclin-dependent kinase inhibitor.

In a further aspect, the invention provides a method for increasing production of a protein by a cell, comprising increasing expression of a *Bcl-x$_L$* gene in the cell, wherein the cell does not express a heterologous cyclin-dependent kinase inhibitor.

In particular embodiments, the cell is a human cell, a murine cell, a hamster cell, an insect cell, or an amphibian cell.

In another aspect, the invention provides a method for increasing the production of a heterologous protein by a cell, comprising increasing expression of a *Bcl-x$_L$* gene in the cell, wherein the cell does not express a heterologous cyclin-dependent kinase inhibitor.

In a further aspect, the invention provides a cell comprising increased expression of an anti-apoptosis gene and does not express a heterologous cyclin-dependent kinase inhibitor, wherein the cell produced an increased amount of a protein as compared to a cell that does not comprise increased expression of the anti-apoptosis gene.

In a further aspect, the invention provides a cell comprising increased expression of a *Bcl-x$_L$* gene and does not express a heterologous cyclin-dependent kinase inhibitor, wherein the cell produced an increased amount of a protein as compared to a cell that does not comprise increased expression of the *Bcl-x$_L$* gene.

In another aspect, the invention provides a cell comprising increased expression of an anti-apoptosis gene and a gene encoding a protein of interest, and does not express a heterologous cyclin-dependent kinase inhibitor, wherein the cell produced an increased amount of a protein of interest as compared to a cell that does not comprise increased expression of the anti-apoptosis gene.

In a further aspect, the invention provides a cell comprising increased expression of a *Bcl-x$_L$* gene and a gene encoding a protein of interest, and does not express a heterologous cyclin-dependent kinase inhibitor, wherein the cell produced an increased amount of a protein of interest as compared to a cell that does not comprise increased expression of the *Bcl-x$_L$* gene.

The invention includes a cell comprising an increased amount of Bcl-x_L protein, where the cell does not express a heterologous cyclin-dependent kinase inhibitor. The cell can be a mammalian, rodent, insect, or amphibian cell, such as a human, murine, or hamster cell (e.g., a Chinese hamster ovary cell). In addition, the cell can be adapted for growth in suspension or for growth in a medium free of serum (e.g., fetal bovine serum). The medium used for culturing the cell, whether free of serum or not, can further contain butyrate (e.g., sodium butyrate) to increase protein yields.

The Bcl-x_L protein can be expressed from an expression vector introduced into the cell or made to overexpress the endogenous *Bcl-x$_L$* gene of the cell, e.g., by inducing the endogenous promoter of the gene. The Bcl-x_L protein can be of a species different than that of the cell. For example, as shown below, the human Bcl-x_L protein can be expressed in Chinese hamster ovary cells to obtain the cells and methods of the invention.

The cells of the invention, as described immediately above, are especially useful for robust production of proteins, either already produced by the cell or exogenously produced by introducing of an expression vector encoding the protein (e.g., a secreted protein). Where the cells of the invention are used to express a cloned monoclonal antibody, the cell can contain one vector that expresses both the heavy and light chain or two vectors, each expressing a heavy or light chain.

Accordingly, the invention further includes a method of producing a polypeptide by culturing a cell of the invention and purifying the polypeptide from the cell culture.

Any publications or other documents cited in this disclosure is hereby incorporated by reference.

Brief Description of the Drawings

Fig. 1A is a schematic representation of the *Bcl-x$_L$* -neo plasmid, a non-limiting vector of the invention. The expression of Bcl-x_L in this vector is driven by the CMV

immediate-early promoter and the neomycin gene provides the selection marker (for resistance in the presence of G418).

Figs. 2A and 2B are schematic representations of growth curves showing viable cell density (VCD) over time (Fig. 2A) and percentage viabilities (% viability) over time (Fig. 2B) for five out of ten non-limiting *Bcl-x_L* transfected Chinese Hamster Ovary (CHO) DG44 cells and two controls (*i.e.*, the untransfected DG44 host and the DG44 transfected with empty vector).

Figs. 3A and 3B are schematic representations of growth curves showing DG44/ Bcl-x_L clone #3, a non-limiting clone of the invention, and two controls (*i.e.*, the untransfected DG44 CHO host and the DG44 CHO cells transfected with empty vector) cultured in the absence of G418 as measured by viable cell density (VCD) over time shown (Fig. 3A) and percentage viability (% viability) (Fig. 3B).

Fig. 4 is a bar graph showing caspase-3 activity, as measured daily for twelve days, in a non-limiting Bcl-x_L transfected cells of the invention, DG44/ Bcl-x_L #3 (black bars), DG44 CHO cells transfected with empty vector (medium gray bars), and untransfected DG44 CHO cells (light gray bars).

Fig. 5 is a representation of a Western blotting analysis probing cell lysates of the following non-limiting cells of the invention: DG44/ Bcl-x_L #3 cells (left lane), DG44/ Bcl-x_L #8 cells (middle lane), and DG44 CHO cells transfected with empty vector (right lane) with a murine monoclonal antibody that specifically binds to human Bcl-x_L protein.

Figs. 6A and 6B are schematic representations of growth curves showing viable cell density (VCD) over time (Fig. 6A) and percentage viabilities (% viability) over time (Fig. 6B) for the following non-limiting cells of the invention: DG44/ Bcl-x_L #3 (black circles), DG44/ Bcl-x_L #8 (blue triangles), and the untransfected DG44 host (open circles).

Fig. 7A is a schematic representation of the *Bcl-x_L* -zeo plasmid, a non-limiting vector of the invention. The expression of Bcl-xL in this vector is driven by the CMV immediate-early promoter and the zeocin gene provides the selection marker (for resistance in the presence of zeocin).

Fig. 7B is a schematic representation of a flow cytometry histogram showing expression of AQC2 by parent 100AB-37 cells (grey [green] line) and the pool of *Bcl-x_L* transfected 100AB-37 cells (bold black [blue] line) as determined by staining with an antibody that specifically binds to AQC2 . The control (black line) was DG44 host cells stained with the same anti-AQC2 antibody

Figs. 8A and 8B are schematic representations of growth curves showing viable cell density (VCD) over time (Fig. 8A) and percentage viabilities (% viability) over time (Fig. 8B) for the following non-limiting cells of the invention: 100AB-37/ Bcl-x_L isolate #11 (purple diamonds), 100AB-37/ Bcl-x_L isolate #21 (black triangles), 100AB-37/ Bcl-x_L isolate #25 (red circles), and 100AB-37 parent (blue circles).

Fig. 9 is a line graph showing the AQC2 titer from for the following non-limiting cells of the invention: 100AB-37/ Bcl-x_L isolate #11 (purple diamonds), 100AB-37/ Bcl-x_L isolate #21 (black triangles), 100AB-37/ Bcl-x_L isolate #25 (red circles), and 100AB-37 parent (blue circles). The 100AB-37/ Bcl-x_L isolates demonstrated significantly higher titers and up to 80% increase in throughput cultured in spinner flasks.

Figs. 10A and 10B are schematic representations of growth curves showing viable cell density (VCD) over time (Fig. 10A) and percentage viabilities (% viability) over time (Fig. 10B) for the following non-limiting cells of the invention: 100AB-37 parent run 1 (open blue diamonds), 100AB-37 parent run 2 (open red squares), 100AB-37/ Bcl-x_L isolate #21, run 1 (black triangles), and 100AB-37/ Bcl-x_L isolate #21, run 2 (red squares) in 2 liter model bioreactors. The results were consistent with previous results obtained in smaller scale spinner cultures.

Fig. 11 is a line graph showing the AQC2 titer from the following non-limiting cells of the invention: 100AB-37 parent run 1 (open triangles), 100AB-37 parent run 2 (open squares), 100AB-37/ Bcl-x_L isolate #21, run 1 (black triangles), and 100AB-37/ Bcl-x_L isolate #21, run 2 (red squares) in 2 liter model bioreactors.

Figs. 12A and 12B are schematic representations of growth curves showing viable cell density (VCD) over time (Fig. 12A) and percentage viabilities (% viability) over time (Fig. 12B) for the following non-limiting cells of the invention: 100AB-37/21.15 Bcl-x_L (green squares) and 37.32 ΔBcl-x_L (open diamonds) cultured in spinners in chemically defined growth media (CDM).

Fig. 13 is a line graph showing the AQC2 titer from the following non-limiting cells of the invention: 100AB-37/21.15 Bcl-x_L (green squares; lead Bcl-x_L-expressing subclone) and 37.32 ΔBcl-x_L (open diamonds; lead subclone of parent) cultured in spinners in chemically defined growth media (CDM).

Fig. 14 is a bar graph showing caspase-3 activity, as measured daily for twelve days, in the following non-limiting cells of the invention: 21.15 Bcl-x_L (red bars; lead Bcl-x_L-expressing subclone) and 37.32 ΔBcl-x_L (gray bars; lead subclone of parent).

Fig. 15 is a bar graph showing the amount of AQC2 secretion by the following non-limiting cells of the invention: 100AB-37 parent cells, 100AB-37.32ΔBcl-x_L cells (lead subclone of parent), 100AB-37-21 Bcl-x_L cells, and 100AB-37-21.15 Bcl-x_L cells (lead Bcl-x_L expressing subclone) in the absence (white bars) or presence (black bars) of 2 mM sodium butyrate in shaker flasks.

Fig. 16 is a bar graph of percent viability for the following non-limiting cells of the invention: 100AB-37 parent cells, 100AB-37.32ΔBcl-x_L cells (lead subclone of parent), 100AB-37.21 Bcl-x_L cells, and 100AB-37-21.15 Bcl-x_L cells (lead Bcl-x_L expressing subclone) in the absence (white bars) or presence (red bars) of 2 mM sodium butyrate in shaker flasks.

Fig. 17 is a bar graph showing caspase-3 activity in the following non-limiting cells of the invention: 100AB-37 parent cells, 100AB-37.32ΔBcl-x_L cells (lead subclone of parent), 100AB-37-21 Bcl-x_L cells, and 100AB-37-21.15 Bcl-x_L cells (lead Bcl-x_L expressing subclone) in the absence (blue bars) or presence (red bars) of 2 mM sodium butyrate in shaker flasks.

Detailed Description of the Preferred Embodiments

The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, published foreign applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference.

The present invention stems from the inventors' unexpected discovery that when an anti-apoptosis gene (*e.g.*, *Bcl-x_L*) is expressed in a cell, that cell produces more protein. Surprisingly, cells co-expressing the anti-apoptosis gene and a second protein (e.g., a heterologous protein) do not show an increase in the number of viable cells. The invention allows for methods to increase protein production by a cell, both *in vitro* (*i.e.*, in tissue culture) and *in vivo*.

Many different genes are involved in the induction and prevention of cell death, including apoptosis and necrosis. Two genes, Bcl-2 and Bcl-x_L have been identified as having anti-apoptosis activities. For example, Fussenegger *et al.* (*Nature Biotechnology* **16(5)**:468-72,1998) describes engineered Chinese Hamster Ovary cells that were engineered to inducibly express three different proteins, Bcl-x_L, p27 (a cyclin-dependent kinase inhibitor), and SEAP (secreted alkaline phosphatase). Upon induction of expression of these proteins, the cells were held in the G1 phase by p27, which allowed an increase in SEAP production. Mastrangelo A.J. *et al.* (*Biotech. Bioeng.* **67(5)**:544-554, 2000) describe induction of cell death of BHK and Chinese Hamster Ovary (CHO) cells by infection with recombinant alphavirus vectors engineered to express IL-12 protein. The lifespans of these infected cells was prolonged by the overexpression of Bcl-2 or Bcl-x_L in these cells, thereby allowing the cells to produce more IL-12. Indeed, overexpression of Bcl-2 or Bcl-x_L in BHK and CHO cells was able to prolong the cells' lifespans after other, non-alphavirus infection induced cell death stimuli, including extended periods of glucose deprivation, serum

withdrawal, and treatment with ammonium chloride (Mastrangelo A.J. *et al.*, *Biotech. Bioeng.* **67(5)**:555-564, 2000).

Thus, as used in accordance with the invention, by "anti-apoptosis gene" is meant the gene encoding the Bcl-2 protein or the gene encoding the Bcl-x_L protein (or other nucleic acid (*e.g.*, cDNA or mRNA) encoding Bcl-2 protein or Bcl-x_L protein, respectively), regardless of what species the genes are from. For example, the *Bcl-x_L* gene may be from a human (GenBank Accession No. Z23115 or L20121; Boise *et al.*, *Cell* **74(4)**: 597-608,1993). Other non-limiting *Bcl-x_L* anti-apoptosis genes of the invention include the feline *Bcl-x_L* gene (GenBank Accession No. AB080951); the bovine *Bcl-x_L* gene (GenBank Accession No. AF245489); the canine *Bcl-x_L* gene (GenBank Accession No. AB073983); the *Xenopus laevis Bcl-x_L* gene (GenBank Accession No. NP_494134); the porcine *Bcl-x_L* gene (GenBank Accession Nos. AF216205 or AJ001203); the murine *Bcl-x_L* genes (GenBank Accession No. U51278, Yang *et al.*, *Immunity* **7(5)**:629-639, 1997; GenBank Accession No. X83574; and GenBank Accession No L35049); and the rat *Bcl-x_L* gene (GenBank Accession No. U34963; Tilly *et al.*, *Endocrinology* **136(1)**: 232-241, 1995).

Similarly, the *Bcl-2* gene may be from a human (GenBank Accession No. M14745; Cleary *et al.*, *Cell* **47(1)**: 19-28, 1986). Other non-limiting anti-apoptosis *Bcl-2* genes of the invention include the rat *Bcl-2* gene (GenBank Accession No. U34964; Tilly *et al.*, *Endocrinology* **136(1)**: 232-241, 1995); the bovine *Bcl-2* gene (GenBank Accession No. U92434); the chicken *Bcl-2* gene (GenBank Accession No. Z11961; Cazals-Hatem *et al.*, *Biochim. Biophys. Acta* **1132(1)**: 109-113, 1992); and murine *Bcl-2* gene (GenBank Accession Nos. NM_009741, M16506, and L31532; Negrini, *Cell* **49(4)**: 455-463, 1987).

In the field of biopharmaceuticals, there are several benefits for delaying the death phase of cultured cells. One such benefit is the opportunity to harvest the product while cell viability is still high thereby reducing the exposure of the product to debris and degradative enzymes produced by cell lysis. Other benefits include reduction in expensive bioreactor runs as a consequence of higher titers, better performance in scale up, simplified down stream processes, and improved cost effectiveness.

In addition, the invention allows the generation of cell lines that may be robust in either chemically defined medium (CDM) or PFM protein free medium (PFM), which, although useful for purifying proteins produced by the cells, are disfavored

since cells grown in protein free media or chemically defined media are highly susceptible to apoptosis. Use of a cell line that is more robust in such media would be highly favorable as this would have an impact on the cost of media, thus eliminating the more expensive (and regulation strict) media components in current formulations.

Accordingly, in one aspect, the invention provides a method for increasing production of a protein by a cell, comprising increasing expression of an anti-apoptosis gene in the cell. In some embodiments, wherein the cell does not express a heterologous cyclin-dependent kinase inhibitor. In particular embodiments, the cell is a human cell, a murine cell, a hamster cell, an insect cell, or an amphibian cell.

In another aspect, the invention provides a method for increasing the production of a heterologous protein by a cell, comprising increasing expression of an anti-apoptosis gene in the cell. In some embodiments, wherein the cell does not express a heterologous cyclin-dependent kinase inhibitor.

Note that as used herein, the term "cell" encompasses all eukaryotic cells including, without limitation, cells from mammals (*e.g.*, human or mouse), insect, amphibian (*e.g.*, *Xenopus laevis*), and birds. Non-limiting examples of cells for use in the invention include CHO cells, NSO cells, BHK cells, NIH-3G3 cells, HEK-293 cells, COS cells, CV1 cells, HeLa cells, Jurkat cells, Raji cells, Daudi cells, Sf9 cells, and A549 cells (all of which are commercially available from the American Type Culture Collection (ATCC), Manassas, VA).

As used herein, by "increasing the expression" is meant that the expression level of an anti-apoptosis gene in a cell is increased as compared to the expression level in the starting cell. For example, where the cell in which the expression of an anti-apoptosis gene does not express any protein encoded by the anti-apoptosis gene (e.g., see the parent CHO DG44 cells described below), any expression of a protein encoded by the anti-apoptosis gene is increasing the expression of that anti-apoptosis gene. Where, however, the parent cell naturally expresses some level of protein encoded by the anti-apoptosis gene, "increasing the expression" of the apoptosis gene results in an increased level of protein as compared to the level expressed by the parent cell.

As used herein, by "expressing" or "expression" is meant that the anti-apoptosis gene is transcribed and/or translated in the cell to produce a protein. For example, where the human *Bcl-x*$_L$ gene is expressed in a murine cell, that murine cell produces human Bcl-x$_L$ protein. Of course, it will be understood that when a cell, in accordance

with the invention, is induced to increase production of a protein by increasing the expression of an anti-apoptosis gene in that cell, the protein the cell is increasing production of is not encoded by the anti-apoptosis gene. For example, if the native *Bcl-x_L* gene is expressed in a murine cell, then, in accordance with the invention, the murine cell that expresses an increased level of its native murine Bcl-x_L protein also increases production of a non-Bcl-x_L protein.

Thus, in accordance with the invention, where a cell of the invention increases production of a protein of interest, that protein can be any protein except for the protein encoded by the anti-apoptosis gene expressed in that cell. The protein can be a secreted protein, a transmembrane protein, or an intracellular protein. Thus, non-limiting examples of proteins include antibodies, hormones (*e.g.*, follicle-stimulating hormone), insulin, nuclear proteins, ribosomal proteins, erythropoietin, cytokines (*e.g.*, interleukin-2 or β-interferon), and blood factors (*e.g.*, Factor IX). The protein can be a native protein to the cell, or can be a heterologous protein to the cell.

As used herein, by the term, "native protein" is meant a protein encoded by a nucleic acid molecule that naturally occurs in the cell. Thus, if the cell is a human cell, a human protein is one that is native to that cell.

As used herein, by the term, "heterologous protein" is meant a protein that is not encoded by a nucleic acid molecule that naturally occurs in the cell. For example, if the cell is a murine cell, a humanized murine antibody is one that is heterologous to that cell. One non-limiting example of a heterologous protein of the invention is the AQC2 antibody, which specifically binds to the cell surface protein, VLA-1 (*e.g.*, human VLA-1). The AQC2 antibody preferably comprises the same heavy and/or light chain sequences as the antibody produced by one of the following hybridoma cell lines, all of which have been deposited with the American Type Culture Collection (Manassas, Virginia, USA) in accordance with the Budapest Treaty: mAQC2 (ATCC Accession No. PTA3273, deposited April 18, 2001); hAQC2 (ATCC Accession No. PTA3275, deposited April 18, 2001); haAQC2 (ATCC Accession No. PTA3356, deposited May 4, 2001); and hsAQC2 (ATCC Accession No. PTA3274, deposited April 18, 2001). These antibodies are described in PCT Publication No. WO02/083854.

Note that the invention encompasses the increased production of an antibody by a cell, including a hybridoma cell. For example, if a hybridoma cell comprises a nucleic acid molecule encoding a particular monoclonal antibody, improved production

of that monoclonal antibody by that cell can be achieved, in accordance with the invention, by expression of an anti-apoptosis gene in that hybridoma cell.

In a non-limiting example, a human B cell is identified as containing nucleic acid that encodes a particular antibody. This B cell is immortalized according to standard methods known in the art (*e.g.*, infection with Epstein Barr virus). Next, to improve the production of the antibody by the now-immortalized B cell, an anti-apoptosis gene can be expressed in the B cell. For example, an expression plasmid encoding an anti-apoptosis gene can be introduced into the cell. Such plasmids are described in the Examples below. Alternatively, the protein encoded by the B cell's own anti-apoptosis gene can be upregulated, such that the native protein is expressed in the cell, thereby resulting in increased production of the antibody.

In accordance with the invention, the cell expressing an anti-apoptosis gene does not express a heterologous cyclin-dependent kinase inhibitor (*i.e.*, a cyclin-dependent kinase inhibitor encoded by a nucleic acid molecule that does not naturally occur in the cell). In certain embodiments, the heterologous cylin-dependent kinase inhibitor is p27. In certain embodiments, the heterologous cylin-dependent kinase inhibitor is p21.

In accordance with the invention, two non-limiting different approaches can be taken for increasing production of a protein. In one approach, a new cell line is generated which has increased expression of an anti-apoptosis gene. As described below, one such non-limiting cell line, Chinese Hamster Ovary cells, was generated. The anti-apoptosis human gene, *Bcl-x_L*, was expressed in these cells, generating a stable Bcl-x_L expressing cell line.

In another non-limiting approach to generate such a cell, the anti-apoptosis gene can also be turned on in a cell that has the gene, but does not express a protein encoded by the gene. For example, a human cell comprises a human *Bcl-x_L* gene, but that does not express human Bcl-x_L, can be induced to express human Bcl-x_L. Such a human cell is included within the scope of the invention.

Once such an anti-apoptosis gene expressing cell line is established, it is ready to produce increased amounts of a protein, regardless of whether that protein is heterologous to the cell or native to the cell. For example, the Bcl-x_L expressing CHO cell may be used to produce increased amounts of a hamster protein. Alternatively, a nucleic acid molecule encoding a heterologous protein (*e.g.*, encoding human β-

interferon) may be introduced (*e.g.*, by transfection, infection, or transformation) into the Bcl-x_L expressing CHO cell, where more heterologous protein is produced by the Bcl-x_L expressing CHO cell as compared to a CHO cell not expressing Bcl-x_L.

Accordingly, in a further aspect, the invention provides a cell comprising increased expression of an anti-apoptosis gene and that does not express a heterologous cyclin dependent kinase inhibitor, wherein the cell produces an increased amount of a protein as compared to a cell that does comprise increased expression the anti-apoptosis gene.

In a second approach of the invention, expression of an anti-apoptosis gene is increased in a cell already producing a protein of interest. In one non-limiting example of the invention, a murine *Bcl-x_L* gene may be expressed in a human β-islet cell (*i.e.*, that already produces human insulin), such that the cell produces more insulin. In another non-limiting example, an anti-apoptosis gene may be expressed in a cell already expressing a heterologous protein (*e.g.*, a CHO cell expressing human β-interferon), such that the cell having increased expression of an anti-apoptosis gene produces more heterologous protein than the cell that does not have an increased expression of the anti-apoptosis gene.

Thus, in a further aspect, the invention provides a cell comprising increased expression of an anti-apoptosis gene and a gene encoding a protein of interest, and does not express a heterologous cyclin-dependent kinase inhibitor, wherein the cell produced an increased amount of a protein of interest as compared to a cell that does not comprise increased expression of the anti-apoptosis gene.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein.

Example I

Generation of an Enhanced CHO Cell Host

To generate a CHO host with improved growth characteristics, which may be potentially used for improved expression of heterologous proteins, Bcl-x_L was expressed in CHO cells.

To do this, the *Bcl-x_L* gene was isolated by using oligonucleotides designed to anneal to the 5' and 3' ends of the open reading frame (ORF) based on the sequence of

Bcl-xL provided in Boise L.H. *et al.*, *Cell* 74: 597-608: 1993 (also see GenBank Accession No. Z23115). The sequences of the oligonucleotides used are as follows: 5' PCR primer 5'-*GCCCTCGAG*ATGTCTCAGAGCAACCGG –3' (SEQ ID NO: 1), where the italicized sequence is an added linker region with an *Xho*I site; and 3'PCR primer 5'-*GCCTCTAGA*TCATTTCCGACTGAAGAGTG –3'(SEQ ID NO: 2), where the italicized sequence is an added linker region with an *Xba*I site

The *Bcl-x_L* gene was generated using the polymerase chain reaction (PCR; using PfuTurbo DNA polymerase, Cat# 600250, commercially available from Stratagene) from Human Brain, whole Marathon-Ready™ cDNA (Clontech Laboratories, Palo Alto, CA). The expression vector, expression vector pcDNA3.1(+) (commercially available form Promega, Madison, WI), was digested with *Xho*I and *Xba*I, and the Bcl-x_L PCR fragment was ligated into the linearized vector. This resulted in the plasmid pBcl-x_L-neo schematically depicted in Fig. 1A.

The pBcl-x_L-neo plasmid was used to transfect CHO-DG44 host cells using electroporation according to standard techniques (see, *e.g.*, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc., New York City, NY, 1993). Note that CHO cells are commercially available from the ATCC. CHO-DG44 cells as described in Urlaub, et al., Cell 33:405-412, 1983. As a control, the empty pcDNA3.1(+) vector was also transfected into CHO cells.

After electroporation, the cells were grown for forty-eight hours in G418-free media, and then selected in the presence of 400 ug/ml G418 (neomycin). The living, adherent cells were selected while the dead, non-adherent cells were removed when the media was changed. After approximately two weeks, stable isolates were selected.

Example II

Improved Growth of the Enhanced CHO Cell Host

The cell death kinetics of these Bcl-x_L transfected cells described in Example I were next compared the to the original unmodified host cells.

To do this, ten DG44/ Bcl-x_L out of fifty isolates, the CHO cell transfected with empty vector were cultured in serum free media supplemented with G418 alongside the untransfected host CHO cell cultured typically in the absence of G418. The cells were cultured for ten days, and were counted daily. For a fed batch mode, cultures were fed every other day with one fiftieth volume of a solution of substrates, without removal of

culture suspension. As shown in Fig. 2A (viable cell density) and Fig. 2B (percentage viability), both controls (*i.e.*, the untransfected DG44 host and the DG44 transfected with empty vector) exhibited maximum VCD on day 4 at approximately 2.6 x 10^6/ml. In comparison, DG44/ Bcl-x_L clones #2, 3, 8, and 9 reached peak VCD on day 5 ranging between 3.0 x 10^6/ml to 3.9 x 10^6/ml, and clone #5 reached a peak of 3.1 x 10^6/ml on day 6. Percentage viabilities for clones #2, 3, and 5 remained high on day 6 at 95, 96, and 87% respectively, whereas viabilities for DG44 (*i.e.*, untransfected host) and DG44 vector alone (*i.e.*, host cells transfected with empty vector) controls had fallen to 59 and 54% respectively. By day 8, viabilities for clones #2, 3, and 5 were at 76, 79, and 81% respectively, whereas viabilities for DG44/neo (*i.e.*, host cells transfected with empty vector) and DG44 controls (*i.e.*, untransfected host) were at 46 and 14% respectively.

The integral cell area (ICA) is defined as the area under a growth profile curve representing the total number of live cells during the course of a culture run. An estimate of the ICA (based on the viable cell density data) on day 8 indicated that isolate #2, 3, 5, and 8 had an increased ICA of 38, 51, 52, and 51%, respectively, over the vector control (*i.e.*, host cells transfected with empty vector). Stability of the previously observed enhanced viability over control cultures for isolates #2, 3, 5, and 8 was repeated and consistent to at least ten passages, (see data for isolate #8 in Figs. 5 and 6). On average the increase in ICA of DG44/ Bcl-x_L cells over the vector only control was 40 to 75%, and was 30 to 100% over the untransfected DG44 host control. Both an increased peak cell density and prolonged cell viability contributed towards the enhanced ICA.

Next, the cells were cultured in the absence of G418 to determine if the presence or absence of G418 had any effect on the growth of the Bcl-x_L transfected cells. To do this, DG44/ Bcl-x_L isolates and control transfected with empty vector alongside the untransfected control, were cultured in the absence of G418. Again, growth curves representing viable cell density (VCD) over time and percentage viability (% viability) over time were monitored.

As shown in Fig. 3A, DG44/ Bcl-x_L clone #3 (one exemplary DG44/ Bcl-x_L isolate) maintained both a higher and prolonged peak cell density of 4 x 10^6 cells/ml up to day 8. Moreover, percent viability was at 90% on day 10 compared to 25% in the vector control (Fig. 3B), and the ICA (approximately 30 x 10^6 cells/ml for a 11-12 day

run) was up to three fold higher than the DG44 host. This characteristic was stable up to at least 7 passages for isolates #2, 3, and 5, but not for #8 (see below).

Example III

Further Characterization of the Enhanced CHO Cell Host

One way to detect and quantify apoptosis is by measurement of caspase-3 proteolytic activity in sample lysates. Caspase-3 is one caspase that plays a critical role in the execution of apoptosis by proteolytic disassembly of cells. Given the known ability of Bcl-x_L to inhibit apoptosis, the CHO cells transfected with Bcl-x_L were next tested for caspase activity using a caspase-3 assay.

Caspase proteins cleave proteins after aspartic acid. It is known that the 3 or 4 amino acids prior to aspartic acid confer specificity. This allows the use of four amino acid labeled peptides to be used as substrates for caspases. For the caspase assay, the peptide substrate used had the amino acid sequence DEVD, with the D (*i.e.*, the aspartic acid residues) labeled with a fluorimetric marker AMC (cat #P-411, BIOMOL Research Labs, Inc., Plymouth Meeting, PA). The marker fluoresces once cleavage has occurred. Thus, without cleavage, little or no signal was observed.

Caspase-3 proteolytic activity was determined from lysates of CHO cells cultured as described in Example II daily for twelve days. Fluorescence of AMC from samples compared to samples treated with non-cleavable analogue DEVD-CHO (BIOMOL cat#P-410), allowed determination of the increase in caspase-3 activity.

DG44/ Bcl-x_L #3 (a non-limiting Bcl-x_L-transfected CHO cell generated according to Examples I and II) showed a delayed onset of peak caspase-3 proteolytic activity as compared to empty vector control cells (*i.e.*, DG44 CHO cells transfected with empty vector) and DG44 CHO control cells (*i.e.*, untransfected cells). As shown in Fig. 4, onset of peak caspase-3 proteolytic activity in DG44/ Bcl-x_L #3 occurred on day 11, with minimal activity exhibited on other days. In contrast the DG44 host alone exhibited over two fold higher peak activity as early as day 5, while the DG44/vector alone control showed close to peak activity starting on day 8 (see Fig. 4). These results demonstrated that genetic manipulation of the host cell line with an anti-apoptosis gene leads to prolonged cell viability.

Next, Western blotting analysis was performed to determine whether or not Bcl-x_L was in fact expressed in DG44/ Bcl-x_L #3. The presence of Bcl-x_L was also assessed

in DG44/ Bcl-x_L #8, a G418 resistant DG44 CHO clone generated at the same time as DG44/ Bcl-x_L #3. To do this, Western blotting was performed according to standard methods (see, *e.g.*, Ausubel *et al.*, *supra*). Briefly, cells lysates were resolved by SDS-Page, and transferred to nitrocellulose or PVDF membranes. The membranes were blotted using a mouse monoclonal antibody that specifically binds to human Bcl-x_L (Clone 2H12, commercially available from Oncogene Research Sciences, San Diego, CA).

As shown in Fig. 5, left lane, Bcl-x_L was clearly expressed in DG44/ Bcl-x_L #3. However, no Bcl-x_L was expressed by either DG44/ Bcl-x_L #8 (middle lane, Fig. 5) or DG44 CHO cells transfected with empty vector (right lane, Fig. 5). The results were the same from isolates grown either in the presence or absence of G418 selection.

Indeed, when DG44/ Bcl-x_L #8 was grown in the absence of G418 for selection, it showed no enhanced survival as compared to untransfected DG44 CHO control cells. To do this, DG44/ Bcl-x_L #8 was released from G418 selection and evaluated against DG44/ Bcl-x_L #3 and the DG44 host control. Growth curves representing viable cell densities (VCD) over time and the percentage viabilities (% viability) were assessed as described in Example I. Although DG44/ Bcl-x_L #8 was G418 resistant with an improved ICA of approximately 50%, when the same cells were cultured in the absence of selection, the increase in ICA was not significant (see Fig. 6A). Moreover, the percent viability over time was not improved and in fact fared worse than the control (see Fig. 6B).

These results demonstrate a correlation between the undetectable Bcl-x_L expression and lack of enhancement in ICA in DG44/ Bcl-x_L #8, as compared to the other isolates such as DG44/ Bcl-x_L #3 in which expression was clearly detected. The results also demonstrate that Bcl-x_L expression is linked to enhanced growth and viability. The observed increase in ICA for DG44/ Bcl-x_L #8 under selective conditions would indicate that the process of G418 enrichment generates cells expressing the neomycin gene (but not always the *Bcl-x_L* gene) with additional robust growth for survival in the presence of G418.

Example IV

Generation of a Bcl-x_L Transfected Cell Line Secreting a Heterologous Protein

The above Examples established the feasibility of generating a more robust CHO host with prolonged cell viability through delay of cell death by expressing the

anti-apoptosis gene, Bcl-x_L, in the cells. To further expand the application of Bcl-x_L, the next goal was to transfect an established CHO-DG44 cell line expressing a heterologous protein with the Bcl-x_L gene and examine the Bcl-x_L transfected cells for an increased production of the heterologous protein arising from expected prolonged viability.

To do this, a second construct was generated as described above in Example I, but with the zeocin resistance gene. Briefly, the Bcl-x_L PCR fragment (see Example I) was cloned into the *Xho*I and *Xba*I sites of expression vector pcDNA3.1/Zeo (+) (Promega, Madison, WI), where expression is driven by the CMV immediate-early promoter and the zeocin gene provides selection marker, to yield final plasmid pBcl-x_L-zeo. A schematic representation of this plasmid is depicted in Fig. 7A.

The pBcl-x_L-zeo plasmid was used to transfect (by electroporation) the cell line 100AB-37, which is a DG44 CHO cell previously transfected with a nucleic acid molecule encoding the monoclonal antibody, AQC2. The 100AB-37 parent secretes the AQC2 monoclonal antibody with a specific productivity (s.p.) of 10 pg cell $^{-1}$ day^{-1}.

The 100AB-37 cells transfected with the Bcl-x_L-zeo plasmid were cultured in the presence of 600 ug/ml zeocin. Next, the pool of transfectants cultured in the absence of fetal bovine serum (FBS) and in the presence of selective zeocin was analysed for AQC2 secretion. Flow cytometry analysis was performed using a conjugated antibody against AQC2. As shown in Fig. 7B, AQC2 secreted from Bcl-x_L transfectants (histogram in bold black) was compared to that of the untransfected parent (grey) and control (black). The results shown in Fig. 7B demonstrated that the ability to express and secrete AQC2 was not suppressed by the presence of Bcl-x_L. Titer analysis by ELISA of conditioned media sampled from the cells above, confirm the flow cytometric data. In fact productivity was higher for Bcl-x_L-transfected cells, at 150 μg/ml for the 100AB-37/ Bcl-x_L pool (2.24 x 10^6/ml cells) compared to 105 μg/ml for the non-modified parent line (2.1 x10^6 cells/ml).

Next, individual isolates of Bcl-x_L transfected 100AB-37 cells were generated, screened for secretion of AQC2, and ranked according to AQC2 titer (*i.e.*, the amount of AQC2 antibody secreted by an isolate). Eight 100AB-37/ Bcl-x_L isolates expressing the highest titer were released from zeocin selection and cultured further for stability before examination for growth and titer (see below) in spinner flasks. As described in Example I, growth curves and % viabilities were monitored as parameters of cell death

kinetics, compared to the parent control, over a period of 14 days in a fed batch mode (as described above). As shown in Fig. 8A, on day 6 the % viability of the parent line was at 95%, which steadily decreased to 62% on day 13, whereas the top three Bcl-x_L isolates (#11, 21, and 25) maintained high % cell viability ranging from 84% to 96%. Interestingly, the sustained higher % viabilities did not result in a significant increase in ICA over the unmodified parent since the overall cell density was not increased (Fig. 8B). Only Bcl-x_L clone #25 showed a moderate 20% increase in ICA (see Fig. 8B).

To assess whether productivity was improved since viability was sustained, the titer of the secreted AQC2 was assayed by protein A-HPLC binding on the eight 100AB-37/ Bcl-x_L isolates evaluated as described above. As many as five out of the eight isolates examined had improved titer ranging from 306 to 434 μg/ml (see below) compared to the parent control of 236 μg/ml on day 12 of culture. As shown in Fig. 9, protein A titer data from clones 11, 21, and 25 shown previously to maintain higher % viabilities (see Figs. 8A and 8B) indicated significant enhancement in productivity.

Protein A titer data on day 14 was as shown below in Table I.

Table I

Cell Line	Day 14 AQC2 (ug/ml)	% increase in throughput	% increase in viability	Specific activity pg/cell/day
100AB-37/ Bcl-x_L #11	368	28	84	14
100AB-37/ Bcl-x_L #21	522	91	95	19
100AB-37/ Bcl-x_L #25	441	53	90	12
100AB-37 parent	289		63	9

(Note that throughput is g/L/day (*i.e.*, the total titer divided by the number of days needed to get to that titer plus two additional days (for bioreactor turnaround time). Thus, percent increase in titer is equivalent to percent increase in throughput).

As Table I demonstrates, 100AB-37/ Bcl-x_L isolates 11, 21, and 25 (% viability above 84%) produced titers of 368, 441, and 522 ug/ml respectively compared to 288 ug/ml from the parent (% viability 62%). The increase in throughput (*i.e.*, titer) of clones ranged from 28% for isolate #11 to as high as 81% for top isolate #21. The specific productivity was also enhanced in the 100AB-37/ Bcl-x_L isolates (12 to 14 pg $cell^{-1}day$-1 compared to 9 pg $cell^{-1}day^{-1}$).

To assess the validity of the previous results demonstrating the marked increase in titer, the evaluation was repeated under the controlled environment of 2 L scale

bioreactors. Bioreactors of this size are typically used to model 200 L manufacture-scale bioreactors. The most desirable 100AB-37/ Bcl-x_L isolate, #21, was run in duplicate reactors along side the 100AB-37 parent. Growth curves and % viabilities were monitored as parameters of cell death kinetics compared to the parent control over a period of 13 days in cltures run in fed-batch mode. Bcl-x_L containing cells were still high in % viabilities at 84 to 89% on day 13, whereas the parent cell line viability had already decreased to 60 and 66% on day 13 (Fig. 10B). As predicted from previous spinner data (see Figs. 8A and 8B), the sustained higher % viabilities did not result in a significant increase in ICA over the unmodified parent since the overall cell density was not increased (FIG. 10A).

Moreover, consistent with small scale spinner data (see Fig. 9), 100AB-37.21/ Bcl-x_L isolate #21 also produced significantly higher titers and up to 60% increase in throughput grown in 2L bioreactors, as determined by protein A binding (Fig. 11).

Protein A titer data on day 14 was as shown below in Table II.

Table II

Cell Line	Day 14 AQC2 (ug/ml)	% increase in throughput	% increase in viability	Specific activity pg/cell/day
100AB-37.21/ Bcl-x_L isolate #21, run 1	597	62	89	21
100AB-37.21/ Bcl-x_L isolate #21. run 2	585	59	84	23
100AB-37 parent, run1	365		66	12
100AB-37 parent, run1	371		60	11

Protein A results indicated that 100AB-37.21/ Bcl-x_L isolate #21 yielded 591 mg/L on day 13 as opposed to only 368 mg/L for the parent line, representing 60% increase in titer and throughput (Fig. 11 and Table II). Significant increase in titer was clearly evident starting on day 7 and 9 and continued throughout the run (see Fig. 11). As previously observed, the ICA from day 0 to 13 was not significantly different; in addition the specific activity was doubled from 11.5 to 22 pg $cell^{-1}$ day^{-1} (Table II).

Furthermore Westerns (non-reduced and reduced SDS-PAGE analysis) and carbohydrate analysis of the AQC2 mAB product from these bioreactor runs indicated no change in product quality.

Example V

Further Characterization of the Bcl-x_L Transfected Cell Line Secreting a Heterologous Protein

Since 100AB-37.21/ Bcl-x_L isolate, #21 was not verified as being clonal, both this isolate along with ΔBcl-x_L 100AB-37 were further subcloned, with the ΔBcl-x_L 100AB-37 isolate being a subclone of the untransfected parent 100AB-37 cell line. This was done because the comparison of the most desirable subclone from each cell line would provide a more strict comparison between Bcl-x_L and ΔBcl-x_L cell lines. To do this, an equivalent number of subclones was screened for each cell line. Specific activity was determined for the selected subclones generated and ranked according to superior growth and ability to produce high titers (data not shown). The lead subclone of Bcl-x_L isolate #21, namely 21.15, was then evaluated and compared to lead subclone 37.32 of the unmodified parent 100AB-37 in spinners vessels. The growth media selected for the evaluation was a chemically defined growth media (CDM) that had few animal derived components. CDM is highly desirable for large scale manufacturing due to the elimination of undefined components, reduction in raw material variability, reduction in complexity of downstream processes and elimination of potential contaminants of animal origin (Jayme and Smith, *Cytotechnology* **33**: 27-36, 2000). Moreover, the CDM environment with markedly reduced protein content is more likely to predispose cells to apoptosis (Moore A. *et al.*, *Cytotechnology* **17**:1-11, 1995 and Zhangi *et al.*, *Biotech Bioeng.* **64**:108-119, 1999). Thus the presence of Bcl-x_L expression may provide a cell line that maintains robustness even under such media conditions.

Growth curves and percent viabilities were monitored as parameters of cell death kinetics compared to the parent control over a period of 14 days in cultures run in fed-batch mode (Fig. 12A). However, as shown in Fig. 12B, 21.15 Bcl-x_L cells sustained high viability well, at above 90% throughout the length of the culture to day 14, whereas cell death occurred in 37.32 ΔBcl-x_L subclone on day 9 (72% viability) and dropped considerably to 43% by day 14. These results demonstrated that Bcl-x_L overexpression delays cell death under media conditions in which cells are particularly susceptible to apoptosis. As expected with previous observations, there was no significant difference in ICA.

Next, to assess the cells' productivity, the titer of the secreted AQC2 was assayed by protein A-HPLC binding. As shown in Fig. 13, lead subclone 100AB-37/21.15 Bcl-x_L produced dramatically higher titers and up to 89% increase in throughput (*i.e.*, titer) compared to lead subclone 37.32 ΔBcl-x_L, even when cultured in chemically defined growth media (CDM).

Protein A titer data on day 14 was as shown below in Table III.

Table III

Cell Line	Day 14 AQC2 (ug/ml)	% increase in throughput	% increase in viability	Specific activity pg/cell/day
21.15 Bcl-x_L	667	89	93	22
37.32 ΔBcl-x_L	353		43	12

As Table III demonstrates, Protein A results of samples taken from cultures described in Figs.12A and 12B indicated that Bcl-x_L 21.15 yielded 667 μg/ml on day 14 as opposed to only 341 μg/ml for the lead subclone of the parent, clone 37.32. This represented as much as 89% increase in throughput when 21.15 cells were still high in percent viability at 93%, compared to 37.32 cells which were low at 43% (Fig. 13 and Table III). Furthermore the specific productivity was almost doubled from 12 to 22 pg cell^{-1}day^{-1} (see Table III). Thus, lead subclone 100AB-37/21.15 Bcl-x_L produced dramatically higher titers and up to 89% increase in throughput compared to lead subclone 37.32 Δ Bcl-x_L, even when cultured in chemically defined growth media (CDM).

To address whether over-expression of Bcl-x_L was responsible for the observed enhancement in titer, assays including the detection and quantitation of Bcl-x_L expression and caspase-3 activity were conducted. Both flow cytometric and Western analysis demonstrated an increase in Bcl-x_L expression from day 3 to a constant level on day 5 in 100AB-37/21.15 Bcl-x_L cells (data not shown). Next, the caspase activity in the 100AB-37/21.15 Bcl-x_L and 37.32 ΔBcl-x_L was assessed. A caspase-3 assay on the cells cultured as described in Fig. 13 (caspase-3 assay performed as described above).

The results showed caspase-3 was dramatically suppressed to control levels throughout the production run of clone 21.15 Bcl-x_L, whereas activity in 37.32 increased almost 10 fold of that of 21.15 on day 14 (see Fig. 14). That the Bcl-x_L

expressing 21.15 Bcl-x_L cells exhibited minimal caspase-3 activity throughout the production run in CDM, demonstrated active suppression of apoptosis. The data clearly indicated significant delay in apoptosis in a cell line that overexpresses Bcl-x_L.

Example VI

Increased Productivity and Viability of Bcl-x_L Transfected Cell Line Secreting a Heterologous Protein Cultured in Media Containing Sodium Butyrate

Sodium butyrate (NaBu) is commonly used as an attempt to enhance specific heterologous protein expression by augmenting transcription (Chang *et al.*, *Free Rad. Es.* **30**:85-91, 1999; Palermo *et al.*, *J. Biotech.* **19**:35-47, 1991; and Laubach, V.E. *et al.*, *Biochem. Biophy. Res. Commun.* **218**:802-807, 1996). However, a serious drawback to the high concentrations of NaBu required for increased expression, is the negative competing effect of rapid induction of apoptosis known to occur in CHO cells (Chang *et al.*, *Free Rad Es* **30**:85-91, 1999; and Kim and Lee, *Biotech. Bioeng.* **71**:184-193, 2000-2001). For this reason NaBu is not universally effective in enhancing titer in all cell lines.

To further analyze the ability of Bcl-x_L to protect cells from premature apoptosis, cells were evaluated up to 4 days with and without 2mM NaBu in shaker flasks cultured in CDM suspension culture. As shown in Fig. 15, additive Na Bu did indeed increase AQC2 productivity of both parent 100AB-37 and the #21 Bcl-x_L expressing isolate, as well as the respective subclones 37.32ΔBcl-x_L, and 21.15.

Moreover, under the conditions described above by day 4, although NaBu increases titer in the parent and 37.32ΔBcl-x_L subclone, the percent viability predictably dropped significantly to 41% and 46% respectively (see Fig. 16). In contrast, % viability for #21 Bcl-x_L remained high at above 80% viability in Bcl-x_L 21.15 cells remained unchanged at 96% (see Fig. 16). These percent viability results demonstrate active suppression of NaBu-induced apoptosis by expression of Bcl-x_L in #21 Bcl-x_L isolate and subclone 21.15 Bcl-x_L.

At day 3 of culture (*i.e.*, in shaker flasks in CDM suspension culture), caspase 3 activity, a marker for apoptosis, was measured in the cells. As shown in Fig. 17, a significant delay in apoptosis was observed in cell lines overexpresing Bcl-x_L. Caspase activity in #21 and 21.15 Bcl-x_L cells was not completely suppressed in the presence of 2 mM butyrate, but was significantly diminished compared to 100AB-37 and 37.32

ΔBcl-x_L, which exhibited 4 fold greater activity (Fig. 17). These results show a positive correlation between Bcl-x_L expression and an active delay of apoptosis chemically induced by NaBu.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.